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PURIFICATION OF THREE ANTIHEMORRHAGIC FACTORS FROM THE SERUM OF A MONGOOSE (*HERPESTES EDWARDSII*)

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Y. TOMIHARA, K. YONAHA, M. NOZAKI, M. YAMAKAWA, T. KAMURA and S. TOYAMA. Purification of three antihemorrhagic factors from the serum of a mongoose *Herpestes edwardsii*. *Toxicon* **25**, 685–689, 1987.—Three antihemorrhagic factors (AHF-1, AHF-2 and AHF-3) were purified from the serum of *H. edwardsii*, a mongoose, by a combination of gel filtration on a Sephadex G-200 column and high performance liquid chromatography with a TSK gel DEAE-5PW column. Each of the purified antihemorrhagic factors showed a single band on polyacrylamide gel disc electrophoresis. The three antihemorrhagic factors inhibited the hemorrhagic activity of HR 1 and HR 2, the hemorrhagic principles from the snake venom of *Trimeresurus flavoviridis* Okinawa. AHF-1, AHF-2 and AHF-3 were stable at temperatures from 0° to 60°C and at pH values between 2.0 and 11.0. The same molecular weight (65,000) was obtained for the three antihemorrhagic factors. No precipitin lines were found for the purified antihemorrhagic factors with the venom of *T. flavoviridis* Okinawa and its hemorrhagic principles, HR 1 and HR 2.

OVADIA and KOCHVA (1977) have shown the resistance of a mongoose, *Herpestes ichneumon*, to some neurotoxins of *Viperidae* and *Elapidae* snakes, but the serum of the warm blooded animal failed to neutralize the toxin. We have found the presence of antihemorrhagic factors in the serum of *H. edwardsii*, a mongoose, against the hemorrhagic principles, HR 1 and HR 2, of *Trimeresurus flavoviridis* Okinawa (Tomihara and Yonaha, unpublished results). In this paper we describe the purification of three antihemorrhagic factors from the serum of *H. edwardsii*.

The serum (1310 mg of protein per 21 ml) obtained from 4 mongooses was applied to a Sephadex G-200 column (3.3 × 70 cm) equilibrated with 5 mM Tris–HCl buffer (pH 8.5) supplemented with 0.15 M NaCl. The column was developed with the same buffer at 4°C. Fractions with antihemorrhagic activity were pooled and concentrated to 10 ml by ultrafiltration with a Millipore membrane filter PSAC under pressure of nitrogen gas. The concentrated fraction was dialyzed against 0.05 M sodium acetate buffer (pH 8.3) at 4°C overnight. The dialysate was subjected to the high performance liquid chromatography (HPLC) on a TSK gel DEAE-5PW column (7.5 × 75 mm) with a Shimadzu LC-4A liquid

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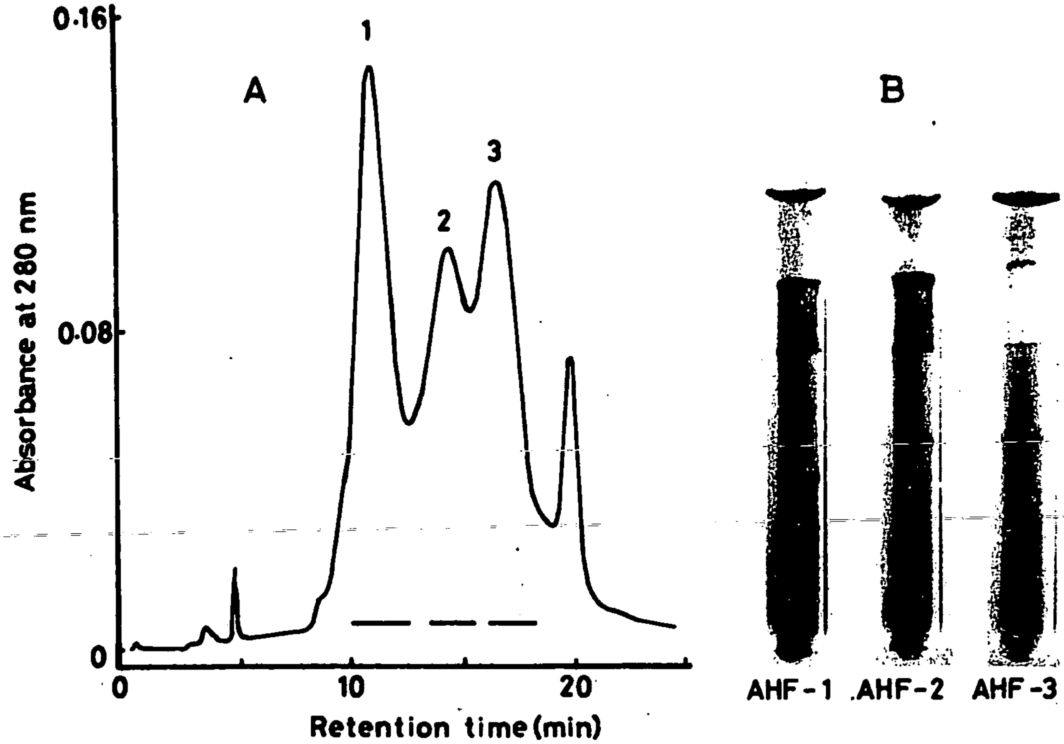

FIG. 1. THIRD HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (A) ON TSK GEL DEAE-5PW AND DISC ELECTROPHORESIS (B) OF THE PURIFIED ANTIHEMORRHAGIC FACTORS OF *Herpestes edwardsii*. (A) The active fractions from the 2nd HPLC were concentrated, dialyzed against 0.01 M potassium phosphate buffer (pH 7.0) and applied to a TSK gel DEAE-5PW column (7.5 × 75 mm). The column was developed with 0.01 M potassium phosphate buffer (pH 7.0, solution A) and the same buffer supplemented with 0.5 M NaCl (solution B) at room temperature. Fractions of 0.5 ml were collected at a flow rate of 1.0 ml/min. After washing the column with 35% B for 5 min, the antihemorrhagic factor was eluted by a linear gradient between 35% B and 60% B for 15 min. Peaks 1, 2 and 3 contained antihemorrhagic factors 1 (AHF-1), 2 (AHF-2) and 3 (AHF-3), respectively. The fractions indicated with a bar were separately pooled. (B) The electrophoresis was carried out under the condition of DAVIS (1964).

chromatography system consisting of a u.v. detector SPD-2AS with variable wavelength and a sample injector SIL-1A with a sample loop of 750 μl. All HPLC operations were performed at room temperature unless otherwise stated. The column was developed with 0.05 M sodium acetate buffer (pH 8.3, solution A) and the same buffer supplemented with 0.25 M NaCl (solution B) and 0.5 ml fractions were collected at a flow rate of 1.0 ml per min. After washing the column with the buffer of 5% B for 5 min, the antihemorrhagic factor was eluted by a linear gradient between 5% B and 80% B for 15 min followed by elution and re-equilibration of the column at 5% B for 5 min. The active fractions were pooled, concentrated by ultrafiltration and dialyzed against 0.05 M sodium acetate buffer (pH 8.3). The dialysate was applied to a column (7.5 × 75 mm) of TSK gel DEAE-5PW and the second HPLC was carried out with the same buffer system as described above. After washing the column with 30% B for 5 min, the antihemorrhagic factor was eluted by a linear gradient between 30% B and 60% B for 10 min followed by elution at 60% B for 4 min and then a linear gradient between 60% B and 70% B for 3

TABLE 1. PURIFICATION OF THREE ANTIHEMORRHAGIC FACTORS FR M THE SERUM OF *Herpestes edwardsii*

Step	Total protein* (mg)	Total activity† (units)	Specific activity† (units/mg)	Yield (%)
Crude serum	1310	19,000	14.4	100.0
Sephadex G-200	460	12,500	27.2	65.8
1st HPLC	70	9,500	135.7	50.0
2nd HPLC	25	8,100	324.0	42.6
3rd HPLC				
AHF-1	5.8	2,500	431.0	13.2
AHF-2	4.8	2,000	416.6	10.5
AHF-3	5.0	1,900	380.0	10.0
4th HPLC				
AHF-1	2.1	2,000	952.4	10.5
AHF-2	1.6	1,600	1000.0	8.4
AHF-3	1.3	1,400	1076.9	7.4

*Protein was determined by absorbance at 280 nm in 1.0 cm light path cuvette, assuming that the absorbance of 1.54 corresponds to 1.0 mg of protein per ml.

†Antihemorrhagic activity was de‹ermined by the method developed by KONDO *et al.* (1960). One unit of activity is defined as the least quantity of protein neutralizing one MHD of the hemorrhagic activity of *Trimeresurus flavoviridis* Okinawa venom. One MHD is defined as the least quantity of venom causing a hemorrhagic spot of 10 mm in diameter 24 hr after intracutaneous injection of venom into the depilated back skin of rabbit. The specific activity is presented as units per mg of protein.

min. The column was re-equilibrated at 30% B for 3 min. The fractions (0.5 ml) were collected at a flow rate of 1.0 ml per min. The fractions with the antihemorrhagic activity were pooled and concentrated as described above. The concentrated fraction was dialyzed against 0.01 M potassium phosphate buffer (pH 7.0) at 4°C overnight and subjected to a third HPLC on a column (7.5 × 75 mm) of TSK gel DEAE-5PW. The column was developed with 0.01 M potassium phosphate buffer (pH 7.0, solution A) and the same buffer supplemented with 0.5 M NaCl (solution B). After washing the column with 35% B for 5 min, the antihemorrhagic factor was eluted by a linear gradient between 35% B and 60% B for 15 min followed by elution and re-equilibration of the column with 35% B for 5 min. By this chromatography the antihemorrhagic activity was separated into three peaks (Fig. 1 A). Peaks 1, 2 and 3 contained antihemorrhagic factors 1 (AHF-1), 2 (AHF-2) and 3 (AHF-3), respectively. Each peak with antihemorrhagic activity was separately pooled and concentrated by ultrafiltration as described above. The concentrated fractions were dialyzed against 5 mM Tris–glycine buffer (pH 8.5) at 4°C overnight and applied separately to a column (7.5 × 75 mm) of TSK gel DEAE-5PW. The fourth HPLC was performed with 5 mM Tris–glycine buffer (pH 8.5, solution A) and the same buffer supplemented with 0.25 M NaCl (solution B). After washing column with 45% B for 5 min, the antihemorrhagic factors were eluted by a linear gradient between 45% B and 80% B for 10 min followed by elution at 80% for 5 min. The column was re-equilibrated with 45% B for 5 min. The fractions (0.5 ml) were collected at a flow rate of 1.0 ml per min. A summary of the purification is presented in Table 1. AHF-1, AHF-2 and AHF-3 were purified to 66.1-fold, 69.4-fold and 74.7-fold in a yield of 10.5%, 8.4% and 7.4%, respectively.

The purified antihemorrhagic factors AHF-1, AHF-2 and AHF-3 gave a single stained protein band on a polyacrylamide gel disc electrophoresis using the conditions of DAVIS (1964) (Fig. 1B). AHF-1 migrated as a more acidic protein than the other two, although AHF-2 and AHF-3 could not be separated on gel electr phoresis. Since OMORI-SATOH *et al.* (1972) have isolated the antihemorrhagic fact r from the serum f *T. flavoviridis*,

several authors have purified antitoxic factors from the serum of snakes (OVADIA *et al.*, 1977; OVADIA, 1978) and mammals (PICHYANGKUL and PEREZ, 1981; MENCHACA and PEREZ, 1981). These authors have found only a single antihemorrhagic factor in the serum of each animal. The three purified antihemorrhagic factors of *H. edwardsii*, therefore, may be the first evidence for the presence of different antihemorrhagic factors against a snake venom in an animal serum. It still remains, however, to be clarified if the difference in the three antihemorrhagic factors is ascribed to a genetic variation in individual mongooses or a modification of protein structure in the course of their purification, e.g. by proteolytic hydrolysis or separate factors. The three purified antihemorrhagic factors neutralized equally the hemorrhagic activities of HR 1 and HR 2, the hemorrhagic principles of *T. flavoviridis* Okinawa which were partially purified by the procedure of OMORI-SATOH *et al.* (1967). The same molecular weight, 65,000, was obtained for the three antihemorrhagic factors by gel filtration on a Sephadex G-200 column as described by ANDREWS (1964). The standard proteins employed were catalase (mol.wt 232,000), aldolase (mol. wt 158,000), bovine serum albumin (mol. wt 68,000) ovalbumin (mol. wt 44,000) and chymotrypsinogen A (mol. wt 25,000). No pricipitin lines were observed for AHF-1, AHF-2 and AHF-3 with the venom of *T. flavoviridis* Okinawa and its hemorrhagic principles HR 1 and HR 2 on double diffusion analysis on an agar gel (OUCHTERLONY, 1949). This agrees with the results obtained with the antihemorrhagic or antineurotoxic factors purified from the sera of various animals, suggesting that the antitoxic factors are not immunoglobulins (OMORI-SATOH *et al.*, 1972; OVADIA *et al.*, 1977; OVADIA, 1978; MENCHACA and PEREZ, 1981; PICHYANGKUL and PEREZ, 1981).

When the purified antihemorrhagic factors were incubated at various temperature for 15 min at pH 7.0, the antihemorrhagic activitities were stable at temperatures from 0° to 60°, though the activities were completely lost at temperatures above 70°. Similar thermal stability was found for the antihemorrhagic factor of *T. flavoviridis* (OMORI-SATOH *et al.*, 1972). The pH stability of the purified antihemorrhagic factors was examined by incubating at room temperature for 1 hr at various pH. The antihemorrhagic factors were characterized by their stability over a wide range of pH; the activities remained unchanged at pH values between 2.0 and 11.0. Similar pH stability was shown for the antihemorrhagic factor of *T. flavoviridis* (OMORI-SATOH *et al.*, 1972), though the factors from other animals were more or less inactivated at extreme pH values (OVADIA, 1978; MENCHACA and PEREZ, 1981; PICHYANGKUL and PEREZ, 1981). No difference was found in the thermal alnd pH stabilities of AHF-1, AHF-2 and AHF-3.

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